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Molecular Mechanism for Loss of Nodulation Properties of *Rhizobium trifolii*

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Of 18 *Rhizobium trifolii* strains tested, 12 showed a high frequency of loss of nodulation ability after incubation in cultures at elevated temperatures. A correlation between loss of nodulation ability and loss of a large plasmid was demonstrated for *R. trifolii*. In some nonnodulating (Nod^-) mutants, deletions occurred instead of total elimination of the plasmid molecule. The maximum curing effect was observed in bacteria incubated at 35°C. After 4 or more days of incubation at this temperature, the viability of bacteria decreased markedly, and the number of nonnodulating mutants increased significantly. At the elevated temperature DNA synthesis was stopped completely after 2 h, whereas protein synthesis proceeded for a few days. Microscopic observations showed that during the first 3 days of incubation at the elevated temperature, the bacterial cells increased markedly in size. These large irregular cells then divided and produced Nod^- clones. Nonnodulating clones did not result from the selection of temperature-resistant mutants. The presence of P-group plasmids in *Rhizobium* strains strongly inhibited the loss of nodulation ability during incubation at 35°C. The observed phenomenon did not result from integrative suppression. It is possible that a product(s) of the genes of R-plasmids acts as a stabilizing agent on the replication process of the indigenous *Rhizobium* plasmids.

The mechanism of infection of plant roots by *Rhizobium* spp. and the development of a symbiotic relationship involving the fixation of atmospheric nitrogen still remain unresolved phenomena. The instability of the symbiotic properties of nodule bacteria has been observed in few laboratories. Spontaneous or induced nonnodulating mutants, as well as mutants with changed nitrogen fixation activity, have been isolated from various species of *Rhizobium* (2). It has been found that after *R. trifolii* strains are incubated at elevated temperatures, they show a high frequency of loss of nodulating ability (23). This phenomenon indirectly suggests that *R. trifolii* nodulation is plasmid controlled. This has been confirmed in further physicochemical and genetic studies (7, 20, 21, 24). The molecular mechanism eliminating the nodulation properties of *R. trifolii* is presented here.

MATERIALS AND METHODS

Bacterial strains. The 18 *R. trifolii* strains presented in Table 1 were used.

Media and cultures. Yeast-mannitol medium (23) was used for bacterial cultivation. The cultures were incubated at 28°C unless otherwise stated.

Temperature curing experiments. Bacteria from the late-exponential growth phase were diluted in fresh medium to a concentration of 10^7 to 10^8 cells per ml and incubated with gentle continuous shaking at a temperature of 32, 35, or 37°C (± 0.2°C). Each day, a sample of each culture was plated onto agar medium, and the plates were incubated at 28°C to obtain single colonies. When solid medium was used in the temperature curing experiments, bacteria from fresh slants were suspended in liquid medium, and 10^5 to 10^8 cells were plated on agar medium or a loopful of bacteria was spread onto a plate. The plates were incubated at 35°C for about 7 days to obtain single colonies.

Plasmid studies. Plasmids were checked by cesium chloride-intercalating dye (ethidium bromide or propidium iodine) density gradient centrifugation and neutral sucrose gradient centrifugation as described previously (24), by gel electrophoresis (5), and by electron microscopy (11).

Determination of protein and DNA syntheses. Protein and DNA syntheses were determined by the measurement of the incorporation of a ^{14}C-labeled amino acid mixture and [6-^3H]thymidine, respectively (both labels were from the Radiochemical Centre, Amersham, England). I measured the level of radioactivity in trichloroacetic acid-precipitated samples of bacterial cultures, using the liquid scintillation method described previously (22).

DNA-DNA hybridization on nitrocellulose filters. Chromosomal DNA of *R. trifolii* 24 was isolated after CsCl buoyant density gradient centrifugation on the basis of the difference between the guanine-plus-cytosine content of chromosomal DNA and that of plasmid DNA (1). Plasmid pWZ1 and pWZ2 DNAs were isolated after preparative neutral sucrose gradient sedimentation (24). Chromosomal and plasmid DNA

preparations after being isolated in this manner were alkali denatured and immobilized on nitrocellulose filters as previously described (9). [^{32}P]DNAs of plasmids pWZ1, pWZ2, and ΔpWZ2 were extracted from agarose gels (8) and used for hybridization with DNA adsorbed onto membrane filters; the formamide technique described by David (6) was employed. The same amount of each type of labeled DNA was used for hybridization with each of the three types of immobilized DNA.

Test of nodulating ability. Nodulating ability was tested on clover seedlings grown in tubes (19). Nodulating *Rhizobium* clones have always given positive results in these tests (thousands of tested plants). At least two plants were used in tests to detect nonnodulating mutants. None of the Nod$^-$ mutants reverted to the Nod$^+$ phenotype (24).

RESULTS

Loss of nodulation ability in *R. trifolii* strains during incubation at elevated temperatures. Standard cultures of *R. trifolii* strains were usually incubated at 28°C. I found that after incubation at elevated temperatures, many strains lost the ability to form nodules on clover: of 18 strains tested, 12 showed a high frequency of loss of nodulation ability after incubation at 35°C (Table 1). After 7 days of incubation at 35°C, up to 80% of the surviving clones of some strains lost the ability to form nodules.

TABLE 1. Loss of *R. trifolii* nodulation ability after incubation at 35°C for 7 days

Source and strain	% of Nod$^-$ mutants in culture[a]
R. Staniewski	
C5	1 (392)
C32	12 (209)
C46	0 (148)
C95	1 (100)
C101	16 (120)
Clover nodules	
T11	0 (115)
T12	63 (469)
T31	80 (382)
T42	7 (117)
28	67 (232)
52	4 (161)
58	9 (173)
W2	54 (463)
E. A. Schwinghamer	
T1	0 (246)
CC2480a	0 (133)
J. M. Vincent	
WD146	0 (202)
WD157	0 (177)
IUNG; 24	75 (950)

[a] Total numbers of tested clones in the cultures are in parentheses.

TABLE 2. Correlation between loss of nodulation ability and plasmid changes in *R. trifolii* strains

Strain	No. of Nod$^-$ mutants tested	Plasmid change of Nod$^-$ mutants	Size of nodulation-conferring plasmid (Mdal)[a]
C5	4	Loss of plasmid pWZ5	112 ± 4 (E)
C32	3	Changes not detected	
T12	5	Loss of plasmid pWZ3 (4 isolates) or deletions in plasmid (1 isolate)	105 ± 6 (EM)
T31	6	Loss of plasmid pWZ7	101 ± 6 (EM)
28	6	Loss of plasmid pWZ6	118 ± 6 (EM)
24	9	Loss of plasmid pWZ2 (7 isolates) or deletions in plasmid (2 isolates)	107 ± 5 (EM)
W2	2	Loss of plasmid pWZ4	103 ± 5 (S)

[a] The sizes of the plasmids were determined by (i) measuring the contour lengths of electron microscopy (EM) pictures of open circular plasmid molecules, using the equivalent of 2.07 Mdal per 1 μm (12); (ii) gel electrophoresis (E); or (iii) neutral sucrose gradient sedimentation (S), using the equation of Böttger et al. (4).

Genetic basis of loss of nodulation ability in *R. trifolii*. I found a correlation between loss of the nodulation phenotype and loss of large plasmids for six of seven strains studied in detail (Table 2). In some Nod$^-$ mutants, small plasmids (ΔpWZ) were detected which were absent in wild-type strains. This fact suggested that in these Nod$^-$ mutants, deletions of up to nearly 70% of the plasmid size occurred instead of the total elimination of the plasmid molecule (Table 2; Fig. 1 through 3). This hypothesis was confirmed by the hybridization data presented in Table 3. The DNA of one small plasmid isolated from a Nod$^-$ mutant of *R. trifolii* 24, ΔpWZ2, showed, as did the DNA of plasmid pWZ2, a high level of hybridization to the filter-immobilized DNA of plasmid pWZ2. In three Nod$^-$ mutants of *R. trifolii* C32, changes in plasmid DNA were not detected by cesium chloride-ethidium bromide density gradient centrifugation and neutral sucrose gradient sedimentation.

Conditions for the isolation of nonnodulating mutants of *R. trifolii*. *R. trifolii* 24 was used for the determination of conditions which cause the loss of nodulation properties. Bacteria from the late-exponential growth phase were diluted in fresh medium and incubated at 32, 35, or 37°C. After incubation at 32°C, Nod$^-$ mutants were not detected in the *R. trifolii* 24 culture (Fig. 4). However, after 4 days of incubation at 35°C, the

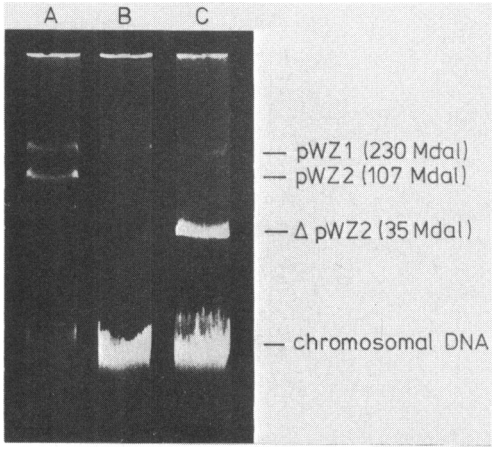

FIG. 1. Agarose gel electrophoresis of plasmid DNA. (A) Plasmid isolated from wild-type nodulating *R. trifolii* 24; (B) plasmid DNA isolated from nonnodulating mutant *R. trifolii* 24(Nod⁻35) obtained after plasmid pWZ2 was cured; (C) plasmid DNA isolated from nonnodulating mutant *R. trifolii* 24(Nod⁻33) (large deletion in plasmid pWZ2).

number of Nod⁻ mutants in cultures increased. About 60% of the cells were Nod⁻ after 7 days of incubation at 35°C. The viability of bacteria at this time rapidly decreased. Incubation at 37°C drastically decreased cell viability, and a maximum of 12% of the cells were Nod⁻ clones. In some strains, a lower level of Nod⁻ mutants was also found after incubation at 32°C. However, the highest frequency of nodulation loss for all six strains occurred at an incubation temperature of 35°C (Table 4). Usually, the increase in number of viable bacteria was inhibited after the incubation temperature was increased to 35°C. However, I found that *R. trifolii* T31 was more resistant than the other strains to elevated temperatures: the number of viable cells of this strain increased twofold after the first day of incubation at 35°C (Fig. 5). In contrast to the results obtained for the other strains, Nod⁻ mutants of strain T31 could be isolated after the first day of incubation at 35°C (Fig. 5B). The optical densities of the cultures of all six *R. trifolii* strains increased at an incubation temperature of 35°C (the data are similar to those in Fig. 5A). Nod⁻ clones did not result from the selection of a temperature-resistant mutant: nearly all of the Nod⁻ clones (>99%) isolated from liquid cultures incubated at 35°C were still sensitive to that temperature. The viability of cells of one Nod⁻ mutant incubated at 35°C is shown in Fig. 6. However, some temperature-resistant mutants could be isolated after incubation at 35°C on agar plates. After 1 week of incubation, small colonies (maximal diameter, ca. 3 mm) appeared on the agar surface (Fig. 7). Most of these colonies (>95%) were stably temperature resistant and could grow well at 35°C (Fig. 6). These mutants appeared in the strain T31 cultures at a frequency of about 10^{-2} and in the cultures of the strains listed in Table 2 at a frequency of 7×10^{-5} to 7×10^{-6}. When bacteria from the colonies growing at 35°C were spread for single clones, it was found that all clones (679 clones tested) obtained from temperature-resistant mutants were nonnodulating.

Macromolecular syntheses in *R. trifolii* cells incubated at an elevated temperature. *R. trifolii* 24 was chosen for the detailed study of protein and DNA syntheses during incubation at an elevated temperature. DNA synthesis was stopped completely after 2 h (Fig. 8A), whereas protein synthesis, although it occurred at a lower rate at 35°C than at 28°C, proceeded for several days (Fig. 8B). In strains T12, C5, and C32, DNA synthesis was also stopped at 35°C after about 2 h of incubation. In the cultures of all of the strains listed in Table 2, the optical density increased during incubation at 35°C (the data are similar to those in Fig. 5A). The cells incubated at 35°C markedly increased in size, which suggested that other macromolecular syntheses, presumably those affecting cell wall synthesis, proceeded at 35°C.

Cytological changes of cells incubated at the elevated temperature. Cells incubated at 35°C markedly increased in size, often to a length of about 10 μm (Fig. 9), after 3 days of incubation. The maximum length of some cells was 20 μm. After 3 to 4 days of incubation at the elevated temperature, these large irregular cells started to divide, and after 5 days, about 60% of the cells were normal sized, a level comparable to that of standard cultures growing at 28°C. All strains listed in Table 2 behaved similarly. The dividing process was correlated to the loss of cell viability (colony-forming ability) and the increased number of Nod⁻ mutants (Fig. 4).

Effect of P-class plasmids on stabilization of nodulation properties of *R. trifolii*. P-class plasmids were introduced to *R. trifolii* from *Pseudomonas aeruginosa* by conjugation, using a crossing method on membrane filters (Millipore Corp.) described previously (21). I found that after the introduction of the P-class plasmid RP4 or R68.45 to *R. trifolii*, the nodulation properties of the strains at elevated temperatures became stable. In the first experiment, freshly isolated strain 24 carrying plasmid RP4 yielded some Nod⁻ mutants (8 of 114 clones tested) after 7 days of incubation at 35°C (Fig. 10). The number of Nod⁻ mutants isolated from the culture of this strain was much lower than that isolated

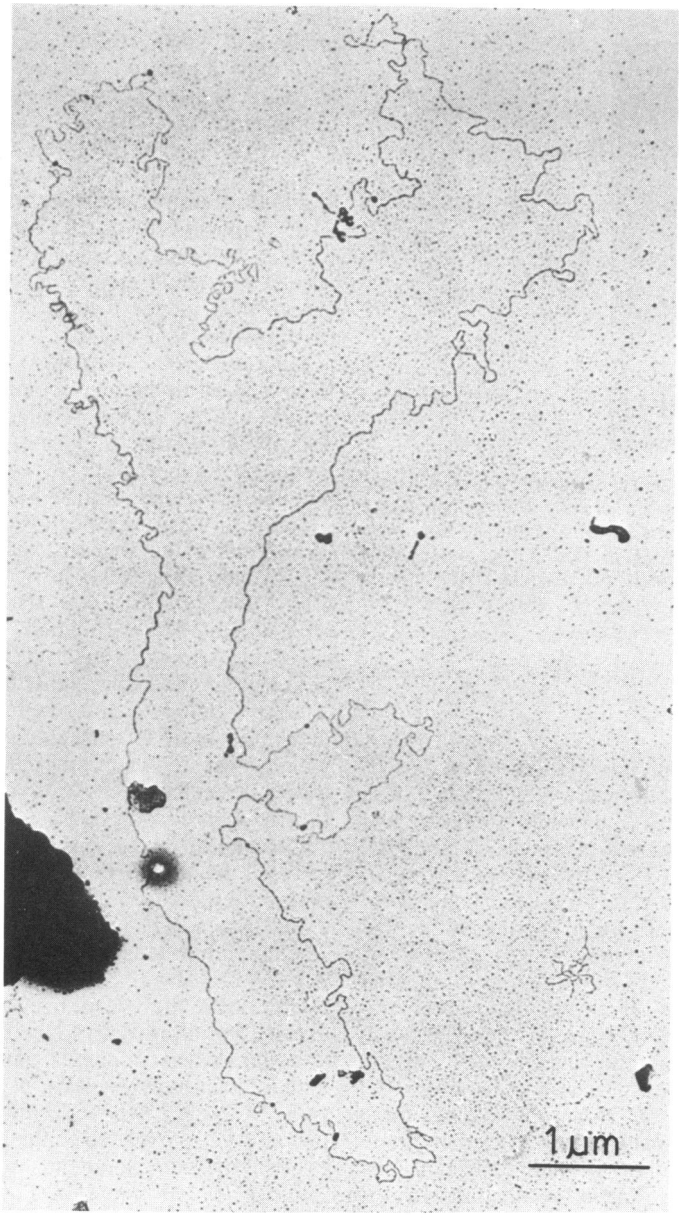

FIG. 2. Electron micrograph of an open circular molecule of the nodulation-conferring plasmid pWZ2, which was isolated from wild-type nodulating *R. trifolii* 24.

from the culture of the wild-type strain. In other experiments Nod^- mutants could not be detected. The total number of tested clones isolated from strain 24(RP4) culture after 7 days of incubation at 35°C was 479. Nod^- clones were never detected (of 318 clones tested) in strain 24(R68.45) cultures incubated at 35°C (Fig. 10). I also found that temperature-resistant clones isolated after incubation of strains 24(RP4) (83 tested clones) and 24(R68.45) (57 tested clones) were still able to nodulate clovers. When cells containing P-class plasmids were incubated at 35°C, they showed the same characteristics as wild-type cells, i.e., inhibited DNA synthesis, lower rate of protein synthesis, and increased optical density, as well as disturbances in the dividing process and changes in cell morphology. The loss of the R-factor after incubation at the elevated temperature was not detected. All of the clones isolated after 6 days of incubation

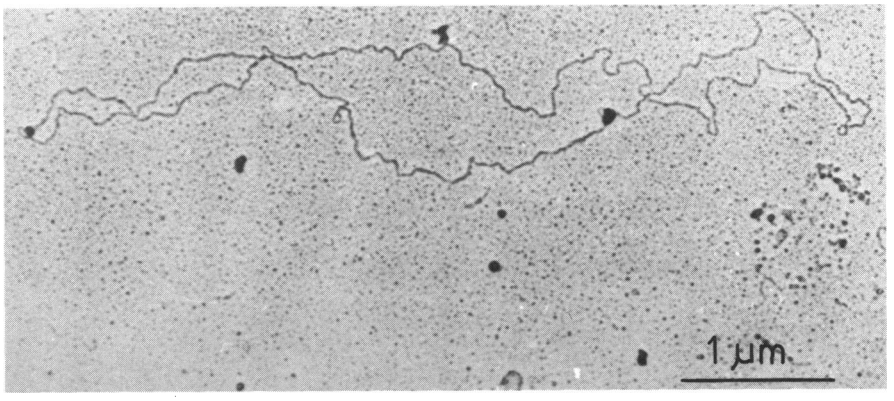

FIG. 3. Electron micrograph of an open circular molecule of deleted plasmid pWZ2, which was isolated from nonnodulating mutant *R. trifolii* 24(Nod⁻33).

of strain 24(RP4) (318 tested clones) and strain 24(R68.45) (401 tested clones) still showed the antibiotic resistance pattern coded by these R-plasmids. In a small number of clones, the loss of some single markers, especially carbenicillin resistance, was detected. However, these losses were not the result of deletions in plasmid molecules: after conjugational transfer to *Escherichia coli*, all of the markers of these R-plasmids were expressed phenotypically. I found no evidence that the integrative suppression was the main mechanism for the stabilization of nodulation properties by R-plasmids. The sucrose gradient sedimentation analysis of plasmid DNA isolated from nodulating clones obtained after incubation of strain 24(RP4) at 35°C showed the presence of plasmid RP4 and two endogenous plasmids of the same size as that found in the original strain 24. When stable Nod⁺ clones containing the RP4 plasmid, isolated after incubation at 35°C, were used in conjugational experiments with a Nod⁻ mutant of strain 24 as a recipient, no cotransfer of nodulation functions and the RP4 factor could be detected (603 transconjugants were tested). It has been shown that the introduction of plasmid pWZ2 restores the nodulation ability of *R. trifolii* 24 (21). It was possible to cure the bacteria of plasmid RP4 by using ethidium bromide; after being cured, the bacteria still nodulated. However, strain 24, after being cured of plasmid RP4 and then incubated at 35°C, lost its nodulation ability at the high frequencies observed for the wild-type strain (Fig. 10).

TABLE 3. Hybridization between different types of DNA isolated from *R. trifolii* 24

DNA immobilized on filter paper	Hybridization of [^{32}P]DNA of[a]:		
	Plasmid pWZ1	Plasmid pWZ2	Plasmid ΔpWZ2
Chromosomal	76 ± 17	35 ± 11	39 ± 12
Plasmid pWZ1	987 ± 89	166 ± 13	172 ± 18
Plasmid pWZ2	259 ± 21	680 ± 67	768 ± 54

[a] Expressed as counts per minute of [^{32}P]DNA binding to the DNA immobilized on filter paper. Each value is the mean result of three experiments.

DISCUSSION

The loss of *Rhizobium* nodulation properties strains has been reported from many labora-

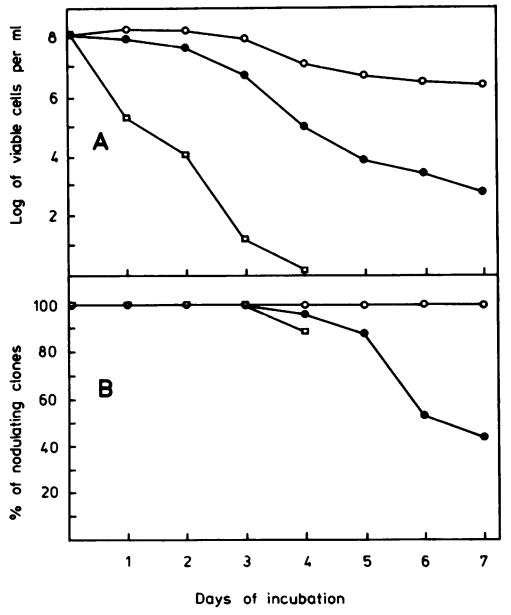

FIG. 4. Effect of incubation at 32°C (○), 35°C (●), or 37°C (□) on *R. trifolii* 24 cell viability (A) and nodulation ability (B).

TABLE 4. Loss of *R. trifolii* nodulation ability after incubation for 6 days at 32 or 35°C or for 3 days at 37°C

Strain	No. of Nod⁻ clones/no. tested		
	32°C	35°C	37°C
24	0/142	51/112	0/89
C5	0/84	6/91	0/72
C32	0/116	16/182	0/196
T12	1/114	54/106	1/134
T31	2/94	63/119	8/101
W2	0/89	56/96	1/147

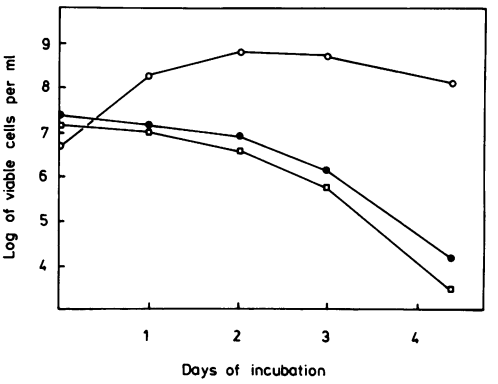

FIG. 6. Viability of nonnodulating mutant *R. trifolii* 24 cells incubated at 35°C. (●) Nod⁻ mutant isolated from a liquid culture; (○) temperature-resistant Nod⁻ mutant isolated from an agar culture; (□), control wild-type strain 24.

tories (2), but in many cases, the nonnodulating bacteria were not tested for the additional characteristics which confirm membership to the genus *Rhizobium*. In this study, many nonnodulating mutants of *R. trifolii* were isolated after incubation at an elevated temperature, and their taxonomic classification was confirmed by physiological, immunological, and genetic tests (7, 17, 20, 21, 23). A high frequency of loss of nodulation properties observed for many *R. trifolii* strains suggested that the nodulation function may be plasmid borne. Studies of *R. trifolii* plasmid DNA have led to the detection of a close correlation between the loss of nodulation ability and the elimination of a plasmid with a molecular mass of 95 to 125 megadaltons (Mdal) (depending on the strain tested). In *R. trifolii* C32, two plasmids with molecular masses of 140 and 240 Mdal have been detected (unpublished data). In three Nod⁻ mutants isolated from cells of *R. trifolii* C32, changes in plasmid pattern were not detected by neutral sucrose gradient centrifugation. However, the high frequency of loss of nodulation observed after incubation of this strain at the elevated temperature suggests that plasmid control of nodulation occurred.

I have obtained genetic results which suggest that incubation of Nod⁻ clones at the elevated temperature induces small deletions (not detectable by comparison of molecular weights of plasmids) in other classes of plasmids detected in *R. trifolii* strains (unpublished data). Large deletions, instead of the often-observed elimination of nodulation-conferring plasmids, were

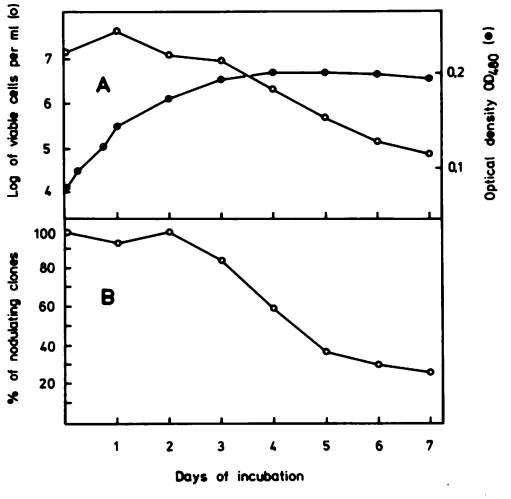

FIG. 5. Effect of incubation at 35°C on *R. trifolii* T31 cell viability and optical density (A) and on nodulation ability (B).

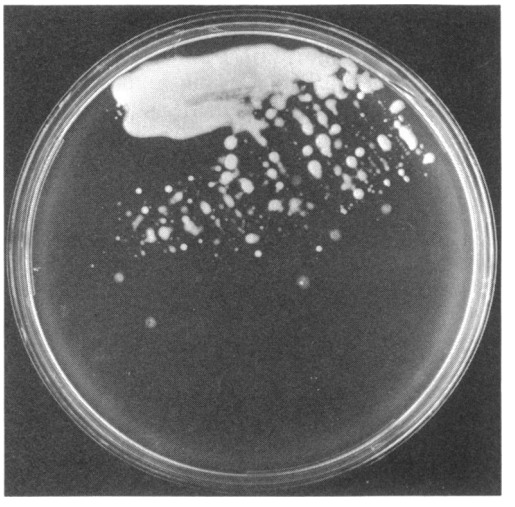

FIG. 7. Temperature-resistant Nod⁻ colonies appeared on agar medium after *R. trifolii* 24 was incubated for 7 days at 35°C.

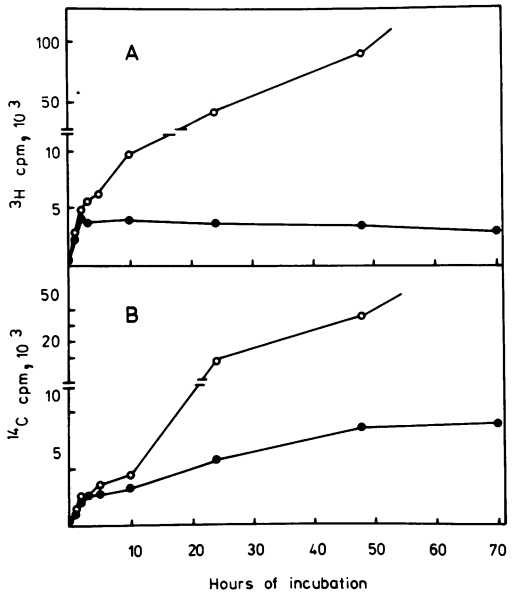

FIG. 8. Incorporation of [^{3}H]thymidine (A) and ^{14}C-labeled amino acids (B) by *R. trifolii* 24 cells incubated at 28°C (○) or 35°C (●).

found in some isolated Nod$^-$ mutants. It is possible, therefore, that in strain C32, small deletions (not detectable by molecular weight comparisons) often occur in the nodulation-conferring plasmid during incubation at the elevated temperature.

The main studies of the temperature-sensitive replication of plasmids have used enterobacteria. Cells of these bacteria usually show good growth and DNA synthesis and a normal dividing process when grown at an elevated temperature (13, 14, 18). *Rhizobium* strains, however, are usually more sensitive to elevated temperatures. At 35°C, growth is markedly inhibited, except for *R. meliloti*, most strains of which can grow well even at >35°C. *R. trifolii* strains incubated at the elevated temperature undergo more complicated changes. The *R. trifolii* process most inhibited by the elevated temperature is DNA synthesis. Aberrant cell division and cell wall synthesis also point to the possibility of the disruption of DNA segregation in cells incubated at the elevated temperature. However, the following facts suggest that the main molecular mechanism for elimination of nodulation ability is the temperature-sensitive replication of the plasmid possessing nodulation functions: (i) the occurrence of deletions in plasmids in some Nod$^-$ mutants, instead of the often-observed elimination of plasmids; (ii) the fast elimination of a plasmid in strain 31 (which grows better at 35°C than do other strains) and in the temperature-resistant *R. trifolii* mutants; (iii) the stabili-

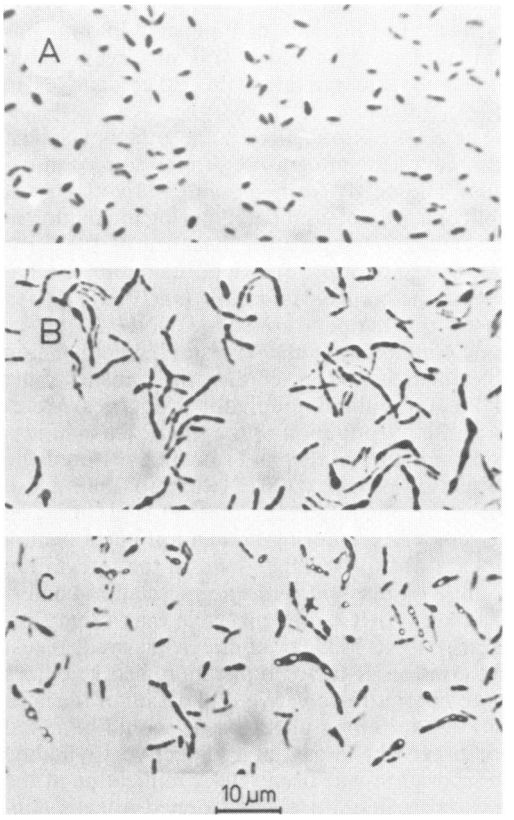

FIG. 9. Phase-contrast microscopy of *R. trifolii* 24 cells incubated under standard growth conditions at 28°C (A), after 3 days of growth at 35°C (B), and after 5 days of growth at 35°C (C). The same magnification was used for all three pictures.

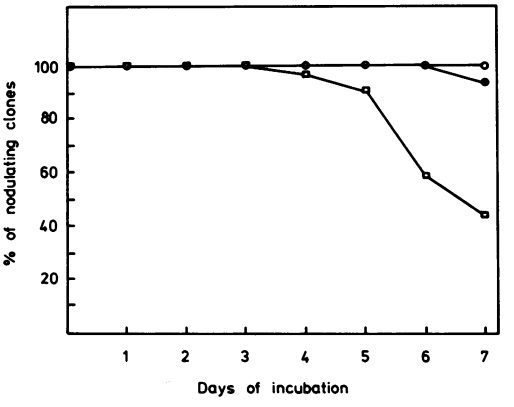

FIG. 10. Effect of P-class R-plasmids on the stability of the nodulation ability of *R. trifolii* strains incubated at 35°C. Data are shown for *R. trifolii* 24 carrying plasmid R68.45 (○) or RP4 (●) and for strain 24 cured of plasmid RP4 (□).

zation of the nodulation phenotype by P-group plasmids; (iv) the lack of P-plasmid loss in cells incubated at 35°C; and (v) the observation that P-plasmids did not alter the other changes in cells incubated at 35°C.

The RP4 plasmid, like some other plasmids, may induce the integrative suppression phenomenon. The newly formed cointegrative forms of replicons are not eliminated during incubation at the elevated temperature because of the RP4-specific temperature-resistant regulation mechanism of the cointegrated replicon (10, 15). When I used the temperature-stable Nod^+ *R. trifolii* clone containing the RP4 plasmid in conjugation experiments, I observed only the transfer of the RP4 factor without nodulation properties. Moreover, the introduction of the pWZ2 plasmid into Nod^- mutants of strain 24 or T12 restored the nodulation property (20, 21; unpublished data). Therefore, the inhibitory effect of R-plasmids on the temperature-curing nodulation phenomenon appears not to be dependent on the integration of the RP4 plasmid with the nodulation-conferring plasmid. It seems probable that a temperature-resistant product of the R-plasmid genes, that controls R-factor replication also stabilizes the temperature-sensitive replication of the nodulation-controlling plasmids. It should be noted that when the assay of radioactive thymidine incorporation was used, DNA replication at the elevated temperature was stopped in cells containing the R-plasmid, as it was in wild-type cells. However, only one additional round of nodulation-controlling plasmid replication during one cell dividing period in 5 days of incubation at an elevated temperature would be sufficient to prevent the elimination of nodulation capacity. Such low levels of DNA synthesis are difficult to measure by this method.

The lack of elimination of the R-plasmids and other endogenous plasmids from *R. trifolii* strains shows that the temperature-sensitive replication of the nodulation-controlling plasmid is conditioned by the properties of the plasmid and not the host cells.

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